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Original Research

Antimicrobial Susceptibility of *Bacteroides* spp. From Clinical Samples From Horses

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ABSTRACT

The objective of the study was to determine the antimicrobial susceptibility of 28 individual clinical isolates from horses, previously identified by phenotypic methods as *Bacteroides* spp., and to species identify these isolates using matrix-assisted-laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and 16S rRNA gene sequencing. Rarely are *Bacteroides* spp. identified to species level, and treatment of infection with anaerobes is seldom guided by antimicrobial susceptibility testing. The gram-negative anaerobic rods were species identified using MALDI-TOF MS and 16S rRNA gene sequencing. Antimicrobial susceptibility was determined by nitrocefin hydrolysis and broth microdilution. The isolates were identified as *Bacteroides fragilis*, *Bacteroides heparinolyticus*, *Bacteroides ovatus*, *Bacteroides pyogenes*, *Bacteroides thetaiotaomicron*, *Bacteroides* sp., *Parabacteroides distasonis*, *Porphyromonas* sp., *Prevotella* sp., and *Prevotella dentasini*. All isolates had low minimum inhibitory concentrations (MICs) of metronidazole. β-Lactamase production and high MICs of penicillin, cephalothin, ceftiofur, erythromycin, fusidic acid, and trimethoprim/sulfamethoxazole were recorded for *B. fragilis*, *B. ovatus*, and *B. thetaiotaomicron*. *Parabacteroides distasonis* did not produce β-lactamase but were still resistant to penicillin and in addition to the aforementioned substances also had high MICs of tetracycline. *Bacteroides heparinolyticus*, *B. pyogenes*, and the isolates identified as *Porphyromonas* sp., *Prevotella* sp., and *P. dentasini* did not produce β-lactamase and were generally more susceptible against the tested antimicrobial agents. It is concluded that antimicrobial susceptibility differs substantially between species of genus *Bacteroides* and it is difficult to species identify anaerobic gram-negative rods by conventional phenotypic methods. It is important both to species identify gram-negative anaerobic rods and perform antimicrobial susceptibility testing to ensure correct treatment.

1. Introduction

Bacteria of genus *Bacteroides* are anaerobic, gram-negative rods. They are important pathogens involved in infections in horses, comprising as much as 8% of anaerobic bacterial infections [1]. Examples of infections involving *Bacteroides* spp. in horses are paraoral and lower respiratory tract infections [2–4]. In one study, it was found that *Bacteroides* spp. made up 20.2% of anaerobes found in cases of pneumonia or pleuropneumoniae [3]. Other

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involvements of *Bacteroides* spp. in horses include endometritis [5], abscesses [6], peritonitis, septic arthritis [4], diarrhea in foals [7], wounds [1,6], and keratitis [8]. Much research has been done on *Bacteroides* spp. in humans (in particular the *B. fragilis* group), but by comparison little in animals. Studies conducted in human medicine identify the *B. fragilis* group as commonly resistant to penicillin and several other antimicrobial agents [9–11]. Often antimicrobial susceptibility results are reported for *Bacteroides* spp. as a group which is unfortunate because different species of *Bacteroides* spp. differ in susceptibility pattern and knowledge about which species of *Bacteroides* that is involved in an infection can aid in the choice of antimicrobial agent. Currently, matrix-assisted-laser-desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) has been introduced as a tool for species identification of bacteria. For many bacterial species, typing with MALDI-TOF MS is rapid and reliable and in particular can improve identification of anaerobic bacteria that can be difficult to type with phenotypic methods [12].

The purpose of this study was to determine the antimicrobial susceptibility of clinical isolates from horses, previously identified by phenotypic methods as *Bacteroides* spp., and to identify the species of these isolates using MALDI-TOF MS or 16S rRNA gene sequencing.

2. Materials and Methods

2.1. Bacterial Isolates

A total of 28 bacterial isolates from clinical submissions to the Department of Bacteriology, National Veterinary Institute (SVA) in Uppsala, Sweden, between 2006 and 2013 were used in this study. The isolates had previously been determined to be of genus *Bacteroides* by different phenotypic methods by veterinary microbiologists and thereafter stored at −70°C. All isolates were from individual equine patients from different clinics in different parts of Sweden. The samples originated from abscesses (n = 7), aspirates from lungs and sinuses (n = 7), wounds (n = 6), peritoneal fluid (n = 3), synovial fluid (n = 2), feces (n = 1), uterus (n = 1), and blood (n = 1). The type strain *B. fragilis* ATCC 25285 was included as a control in the antimicrobial susceptibility tests.

Growth of *Bacteroides* was considered to be of clinical significance in all samples except of the fecal sample. Anaerobic bacterial isolates were typed and considered as significant if growth was in pure culture or dominating on the agar plate. Conventional methods for isolation and identification of microorganisms were used [13].

2.2. Bacterial Culture and Species Identification

Thawed isolates were cultured on fastidious anaerobe agar (FAA) supplemented with 10% horse blood (National Veterinary Institute, Uppsala, Sweden) and incubated for 48 hours at 37°C in a 2.5-L anaerobic jar. The anaerobic environment was created using Oxoid AnaeroGen sachets (Oxoid Ltd, Basingstoke, UK). The samples were then recultured twice on FAA and incubated at 37°C for 48 hours before the susceptibility test to ensure vitality of the bacteria. All isolates were analyzed by the MALDI Biotyper system (Bruker Daltonics, Bremen, Germany) to identify the species. Mass spectra were compared against 4613 spectra in the MALDI Biotyper database using the MALDI Biotyper 3.0 real-time classification software (Bruker Daltonics). Material from a single colony from the FAA plate was spotted on a MALDI plate without pretreatment. The spots were covered with 1 μL matrix solution consisting of α-cyano-4-hydroxycinnamic acid and air-dried in room temperature after which it was introduced into the MALDI-TOF mass spectrometer for analysis. The spectra of all isolates were compared to the spectra in the database, and identification was provided with a score of reliability. A score ≥2.0 was considered as reliable species identification. A score-oriented dendrogram constructed from main spectra projections (MSPs) of the generated spectra was created in MALDI Biotyper 3.1 with the Biotyper MSP Dendrogram Creation Standard Method default settings (distance measure: correlation and linkage: average).

For isolates that MALDI-TOF MS could not provide reliable results or could not provide any results because the spectra did not exist in the database, the identification was carried out using 16S rRNA gene sequencing. A polymerase chain reaction (PCR) was performed using the bacteria-specific primer Bac27 F (5′-AGAGTTTGGATCMTGGCT CAG-3′) and the universal primer Univ1492 R (5′-CG GTTACCTTGTTACGACTT-3′). A 50 μL reaction mixture with Qiagen HotStarTaq Master Mix (Qiagen, Hilden, Germany), 0.2 μM each of the primers, and 2 μL of boiled lysate from the isolates was prepared. The thermal cycling conditions were 95°C for 15 minutes, 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 90seconds; and a final extension at 72°C for 10 minutes. Before sequencing, the PCR product was treated enzymatically using FastAP Thermosensitive Alkaline Phosphatase (Thermo Scientific, Waltham, MA) and Exonuclease I *E.coli* (Thermo Scientific). For the sequencing reaction, the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) was used. Sequencing was performed on an ABI PRISM 3,100 Genetic Analyzer (Applied Biosystems) according to the manufacturer's protocol.

For sequence editing and analysis, CLC Main Workbench 6 (CLC bio) was used and for homology searches the BLAST algorithm [14] at the National Center for Biotechnology Information.

2.3. Antimicrobial Susceptibility Testing

All isolates were tested for antimicrobial susceptibility using VetMIC GP-mo (ver. 2), VetMIC small animals (ver. 2), and VetMIC CLIN GN microdilution panels (SVA, Uppsala, Sweden). An additional panel containing only metronidazole was designed especially for this study. Isolates were tested by broth microdilution in Brucella broth supplemented with hemin (5 μg/mL), vitamin K_1 (1 μg/mL), and lysed horse blood (5%) according to the Clinical and Laboratory Standards Institute's (CLSI) standard for antimicrobial susceptibility testing for anaerobic bacteria [15]. Reading of minimum inhibitory concentration (MIC) was performed using a BIOMIC V3 96-well microdilution plate reader (Giles Scientific, Santa Barbara, CA). The MIC was